PAS Kinase Is a Nutrient and Energy Sensor in Hypothalamic Areas Required for the Normal Function of AMPK and mTOR/S6K1

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Abstract: The complications caused by overweight, obesity and type 2 diabetes are one of the main problems that increase morbidity and mortality in developed countries. Hypothalamic metabolic sensors play an important role in the control of feeding and energy homeostasis. PAS kinase (PASK) is a nutrient sensor proposed as a regulator of glucose metabolism and cellular energy. The role of PASK might be similar to other known metabolic sensors, such as AMP-activated protein kinase (AMPK) and the mammalian target of rapamycin (mTOR). PASK-deficient mice resist diet-induced obesity.We have recently reported that AMPK and mTOR/S6K1 pathways are regulated in the ventromedial and lateral hypothalamus in response to nutritional states, being modulated by anorexigenic glucagon-like peptide-1 (GLP-1)/exendin-4 in lean and obese rats. We identified PASK in hypothalamic areas, and its expression was regulated under fasting/refeeding conditions and modulated by exendin-4. Furthermore, PASK-deficient mice have an impaired activation response of AMPK and mTOR/S6K1 pathways. Thus, hypothalamic AMPK and S6K1 were highly activated under fasted/re-fed conditions. Additionally, in this study, we have observed that the exendin-4 regulatory effect in the activity of metabolic sensors was lost in PASK-deficient mice, and the anorexigenic properties of exendin-4 were significantly reduced, suggesting that PASK could be a mediator in the GLP-1 signalling pathway. Our data indicated that the PASK function could be critical for preserving the nutrient effect on AMPK and mTOR/S6K1 pathways and maintain the regulatory role of exendin-4 in food intake. Some of the antidiabetogenic effects of exendin-4 might be modulated through these processes.

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PAS Kinase Is a Nutrient and Energy Sensor in Hypothalamic Areas Required for the Normal Function of AMPK and mTOR/S6K1

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Keywords AMPK · Antidiabetogenic agents · Food intake · Metabolic sensors · mTOR/S6K · PASK · Obesity

Introduction

The PAS kinase (PASK), also termed PASKIN, is a nutrient sensor conserved from yeast to human beings. In mammals, PASK is involved in the regulation of glucose and energy metabolism homeostasis [1–3]. Thus, it has been proposed that PASK is activated by small metabolites and may regulate glycogen synthesis and protein translation. PASK could regulate glucose metabolism and cellular energy homeostasis [4, 5]. Initially, PASK-deficient mice showed no developmental, growth, or reproductive defects [6]. Later investigation indicated that PASK-deficient mice are resistant to diet-induced obesity, and PASK knockdown by RNAi in cultured myoblasts causes increased glucose and palmitate oxidation and elevated cellular ATP levels [7]. Additionally, PASK is also implicated in the regulation of glucagon secretion by glucose, and PASK expression was lower in pancreatic islets from human with type 2 diabetes [8]. The role of PASK might therefore be similar to better known metabolic sensors, such as AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR). AMPK senses cellular energy availability by detecting the AMP/ATP ratio. AMPK is activated in low energy states and promotes ATP-generating catabolic pathways and inhibits anabolic reactions [9–11]. By contrast, mTOR and its downstream target, the S6 kinase 1 (S6K1), respond to nutrients and hormonal signals [12–14] and increase their activities in the presence of nutrient abundance [15]. Thus, mTOR complex 1 (mTORC1) is rapamycin- and nutrient-sensitive, regulating metabolism and cell growth in response to several environmental signals. Accordingly, amino acids, growth factors and mitogens activate mTORC1 and lead to an increase in protein synthesis and stimulation of cell growth [16]. By contrast, mTORC1 activity is inhibited under conditions of energy depletion coordinated with AMPK activity. An increase in the AMP/ATP ratio activates AMPK, which phosphorylates the tuberous sclerosis complex 2 (TSC2), and this modification induces the concomitant inhibition of mTORC1 mediated by TSC1-TSC2 complex [17]. Furthermore, AMPK phosphorylates raptor (rapamycin-sensitive adaptor protein of mTOR) in mTORC1, inducing the downregulation of this complex [18]. Additionally, energy depletion inhibits mTORC1 through an independent mechanism of AMPK activation [19]. AMPK is additionally inhibited by the phosphorylation of mTOR/S6K1 [20]. The hypothalamic AMPK [21] and mTOR/S6K pathways [22] have been implicated in the control of feeding and the regulation of energy balances.

We have recently reported that PASK expression is regulated by glucose and glucagon-like peptide-1 (GLP-1) in rat ventromedial (VMH) and lateral hypothalamus (LH) areas from hypothalamic organotypic cultures and that there is an impaired response of AMPK and mTOR/S6K1 pathways in PASK-silenced neuroblastoma cells [23]. GLP-1 is an incretin, resulting from the posttranslational processing of preproglucagon that is secreted by L-intestinal cells after eating. Its structural analogue, exendin-4, is used as an antidiabetogenic agent due to its longer half-life and ability to promote the secretion of insulin when glucose increases. In addition, exendin-4 has anorexigenic properties administered both peripheral and at the central nervous system level [24]. In this sense, we have recently reported that the regulation of AMPK and mTOR/S6K1 pathways in response to changes in nutrient concentration could be modulated by the presence of GLP-1/exendin-4, which counteracts the activation/inactivation of AMPK and mTOR/S6K1 in the hypothalamic areas involved in feeding behaviour (VMH and LH) [25]. Thus, it seems that exendin-4 might balance the proper activation of these metabolic sensors in lean and obese rats, helping to improve pathophysiological states such as obesity [25].

Here, we have investigated the expression of PASK in mouse brain, as well as the effects of nutrients and exendin-4 on its expression in hypothalamic areas involved in feeding behaviour. Furthermore, we have investigated the possible relationships between PASK and other metabolic sensors by analysing the expression and activity of AMPK and mTOR/S6K1 pathways in VMH and LH areas under fasting and re-feeding conditions, as well as the effect of the anorexigenic peptide exendin-4 in PASK-deficient mice.

Materials and Methods

Animals

All procedures involving animals were approved by the appropriate Institutional Review Committee and met the guidelines for the care of animals specified by the European Community. The animals used were 10- to 16-week-old males (25–30 g), C57Bl/6 wild-type and PASK-deficient mice backcrossed into C57Bl/6 for at least 12 generations [6]. The animals were fed ad libitum with a standard pellet diet and housed at a constant temperature (21 °C) on a 12-h light-dark cycle, with lights on at 8 a.m.

Measuring Blood Plasma Glucose and Insulin Levels

Glucose levels were sampled from mouse tail bleeds using a Glucometer Elite meter (Bayer Corp., Elkhart, IN, USA).

Blood plasma insulin levels were determined using a competitive ELISA Kit (Millipore, MA, USA), following the manufacturer's instructions.

X-Gal Staining

Hypothalamic slices of 500 μm were used for 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-Gal) staining. Hypothalamic slices were fixed in 0.2 % glutaraldehyde, 5 mM EGTA, 2 mM $MgCl_2$ and 0.1 M Na-phosphate buffer (pH 7.3) for 16 h. Then, they were permeabilized in 0.01 % Na-deoxycholate, 0.02 % NP-40, 2 mM $MgCl_2$ and 0.1 M Na-phosphate buffer (pH 7.3) and incubated in X-Gal solution [5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 0.02 % NP-40, 0.01 % Na-deoxycholate, 0.1 M Na-phosphate buffer (pH 7.3), 0.1 % X-Gal] for at least 5 h at 30 °C until colour developed.

Isolation of Hypothalamic Areas

Mice *Pask*$^{-/-}$ and C57Bl/6 wild-type mice were fasted for 48 h. Some animals were re-fed for 3 h, and some of them were treated subcutaneously with exendin-4 (250 ng/100 g body weight, Bachem) for 1 to 4 h. The hypothalami were removed from the brains and sectioned at 500 μm. VMH and LH were isolated by micropunching according to the stereotaxic coordinates [26, 27].

Polymerase Chain Reaction

The total RNA from VMH and LH from C57Bl/6 wild-type and PASK-deficient mice was isolated by the acid-guanidinium isothiocyanate method [28]. RNA integrity was tested with the Bioanalyzer 2100 (Agilent), and cDNA synthesis was developed using the High-Capacity cDNA Archive Kit (Applied Biosystems), using 1 μg of RNA as a template, following the manufacturer's instructions. In order to ensure the purity of VMH and LH, a PCR was developed using the primers to amplify *steroidogenic factor 1* (*Sf-1*) and *prepro-orexin* (Online resource 1) as described [23]. Briefly, the PCR conditions were as follows: 94 °C for 2 min, followed by 30 cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min, and a final extension cycle at 72 °C for 10 min. The amplified fragments were analysed by agarose gel electrophoresis.

Real-time polymerase chain reaction (TaqMan® Assay). The mRNA levels of *Pask*, *Ampkα2*, *Glp-1r*, *β-actin* and *18s rRNA* were measured by real-time quantitative RT-PCR using TaqMan® probes (Applied Biosystems) (Online resource 1) as described [23]. Briefly, PCR was developed in a 7900HT Fast Real-Time PCR System (Applied Biosystems). The conditions were 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min.

Multiple gene expression analyses in TaqMan® Gene Expression Assay. Quantity real-time PCR was developed using the TaqMan® Fast Universal PCR Master Mix and Roche Diagnostics on TaqMan® Array 96-Well Fast Plates containing 17 customized duplicated genes (Online resource 1) as described [23]. Briefly, 2 μl of the synthesized cDNA was diluted in a final volume of 100 μl and applied to each port of the TLDA card. PCR was developed in a 7900HT Fast Real-Time PCR System (Applied Biosystems). The conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. *18s* and *β-actin* housekeeping genes were used for normalization. Median normalization was performed as follows: the median cycle threshold (CT) value of all the genes on each TLDA card was subtracted from the CT value for each reaction well on that card, whereby after normalization, the median CT value on each card was zero. The average *18s* or *β-actin* CT values on each TLDA card were then subtracted from the CT value for each reaction well on that card.

Protein Expression and Activity of AMPK and mTOR/S6K1 Pathways

For the analysis of protein expression and activity by western blotting, a tiny piece of frozen liver (~150 mg) or the hypothalamic nuclei were immediately lysed in RIPA buffer [150 mM NaCl, 50 mM Tris-HCl (pH 7.2), 1 % sodium deoxycholate, 1 % Triton X-100, 0.25 mM EDTA (pH 8.0), 10 mM NaF, 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate] and a tablet of protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Tissues were then exposed immediately to microwave irradiation for 5 s and then homogenized [25]. Total and phosphorylated forms were detected by western blotting using the antibodies described (Table 1). The blots were reprobed for β-actin. Finally, the blots were scanned and quantified using Quantity One software (Bio-Rad, GS-800 Densitometer).

Food Intake

The feeding response to exendin-4 treatment was tested as previously described [29]. Briefly, male C57Bl/6 wild-type and PASK-deficient mice were fasted overnight. Exendin-4 (250 ng/100 g body weight, Bachem) in saline or the saline vehicle alone was subcutaneously injected, and 1 h after the exendin injection, the mice were re-fed and the food intake was measured 30, 60 and 120 min later.

Statistical Analyses

All values are presented as means ± SEM. The groups were compared using a *t* test. $P<0.05$ was considered statistically significant.

Table 1 Antibodies and conditions used for western blot assays

Antibody	Host	Manufacturer	Dilution used
Anti-ACC	Rabbit	Millipore Iberica, Madrid, Spain	1:1,000
Anti-phospho-ACC(Ser 79)	Rabbit	Millipore Iberica, Madrid, Spain	1:500
Anti-AMPKα	Rabbit	Cell Signaling, Danvers, MA, USA	1:1,000
Anti-phospho-AMPKα(Thr172)	Rabbit	Cell Signaling, Danvers, MA, USA	1:1,000
Anti-β-actin	Mouse	Sigma-Aldrich, Saint Louis, Missouri, USA	1:5,000
Anti-S6K1	Rabbit	Cell Signaling, Danvers, MA, USA	1:1,000
Anti-phospho-S6K1(Thr389)	Rabbit	Cell Signaling, Danvers, MA, USA	1:1,000
Anti-rabbit-HRP	Goat	Millipore Iberica, Madrid, Spain	1:5,000
Anti-mouse-HRP	Goat	Bethyl Laboratories, Montgomery, USA	1:5,000

Results

PASK Expression in Mouse Hypothalamus

X-Gal staining of hypothalamic region of *Pask*$^{-/-}$ mice revealed PASK-positive cells located in several hypothalamic areas, mainly in arcuate and paraventricular nuclei, as well as in VMH and less intensively in LH. A strong labelling was also observed in the walls lining the third ventricle (Fig. 1a). *Pask* mRNA was detected using two sets of primers (Fig. 1b). No detection of the *Pask* mRNA in PASK-deficient mice was observed using primers that recognize the kinase domain, as is to be expected in *Pask*$^{-/-}$ mice. However, primers recognizing exons 7 and 8, which are maintained in PASK-deficient mice, recorded a higher level of mRNA in both VMH and LH in *Pask*$^{-/-}$ mice compared to wild-type specimens (Fig. 1b, c).

Effect of PASK Deficiency on the Expression of Metabolic Genes in Mouse VMH and LH

To evaluate the role that PASK plays in the control of feeding, we analysed the expression of other metabolic sensors in hypothalamic areas involved in food intake and energy homeostasis. The mRNA levels were measured with real-time RT-PCR in VMH and LH from fasted C57Bl/6 wild-type and PASK-knockout mice in the presence or absence of exendin-4. We measured the expression of genes coding for metabolic sensors including AMPKα2 and mTOR/S6K1; several genes as direct or indirect regulators of metabolic sensor activity, such as AKT, LKB1, PKARIIα and TSC2; several direct substrates of PASK, AMPK and mTOR/S6K1 pathways: S6, eIF4B, eEF-2K, eEF1-α1 and nNOS; genes whose products regulate carbohydrate metabolism: GAPDH and PDK1; and additionally, GLP-1 receptor (GLP-1R), homeodomain-interacting protein kinase 2 (HIPK2), a mediator of apoptotic pathways in neurons and a housekeeping gene: *β-actin*. In the absence of any treatment, the expression of mRNA coding for mTOR, AMPKα2 and S6K1 was similar in VMH and LH from wild-type and PASK-deficient mice, but PASK-deficient mice had lower *Ampkα2* mRNA levels in VMH, and a similar tendency was observed in LH, although no significant differences were found (Fig. 2a). The expression of genes coding for AKT, LKB1, PKARIIα and TSC2 was similar in PASK-deficient and wild-type mice (Fig. 2b); our data also showed no changes in the expression of mRNAs coding for S6, eEF1-α1, eEF-2K, eIF4B and nNOS1 (Online resource 2A), and similar expressions of GADPH and PDK1 (Online resource 2B) were found in both types of mice. The expression of genes coding for PKARIIα, eIF4B, eEF1-α1 and nNOS1 was lower in LH than VMH in wild-type mice. By contrast, *Hipk2* mRNA levels were higher in LH from PASK-deficient mice (Fig. 2c). Therefore, our data suggests that the expression of *Ampkα2* and *Hipk2* (a mediator of apoptotic pathways in neurons) might be mediated by PASK protein either directly or indirectly. After exendin-4 treatment, we observed a decreased expression of GLP-1R in VMH and LH from wild-type mice (Fig. 2c). By contrast, the presence of exendin-4 increased the expression of LKB1, eEF1-α1, and S6 in VMH from wild-type mice (Fig. 2b and Online resource 2A). However, the absence of an exendin-4 effect in PASK-deficient mice explains the lower levels of LKB1, TSC2, AKT1, mTOR, eEF1-α1 and nNOS1 in VMH from PASK-deficient mice compared to their wild-type counterparts.

PASK Expression Responds to Fasting and Re-feeding in VMH and LH from Wild-Type Mice

PASK has already been proposed as a nutrient sensor [7], so we analysed PASK mRNA expression under fasting and re-feeding conditions in VMH and LH from wild-type and PASK-deficient mice. In order to ensure the purity of these hypothalamic nuclei obtained by micropunching from mouse hypothalamus, *Sf-1* and *prepro-orexin* genes were used as markers of these specific nuclei, respectively. The mRNA encoding SF-1 was mostly present in VMH, whereas *prepro-orexin* mRNA levels were higher in LH (Fig. 3a). Re-feeding increased *Pask* mRNA expression in LH in wild-type mice. The *Pask* mRNA level was lower in LH than that in

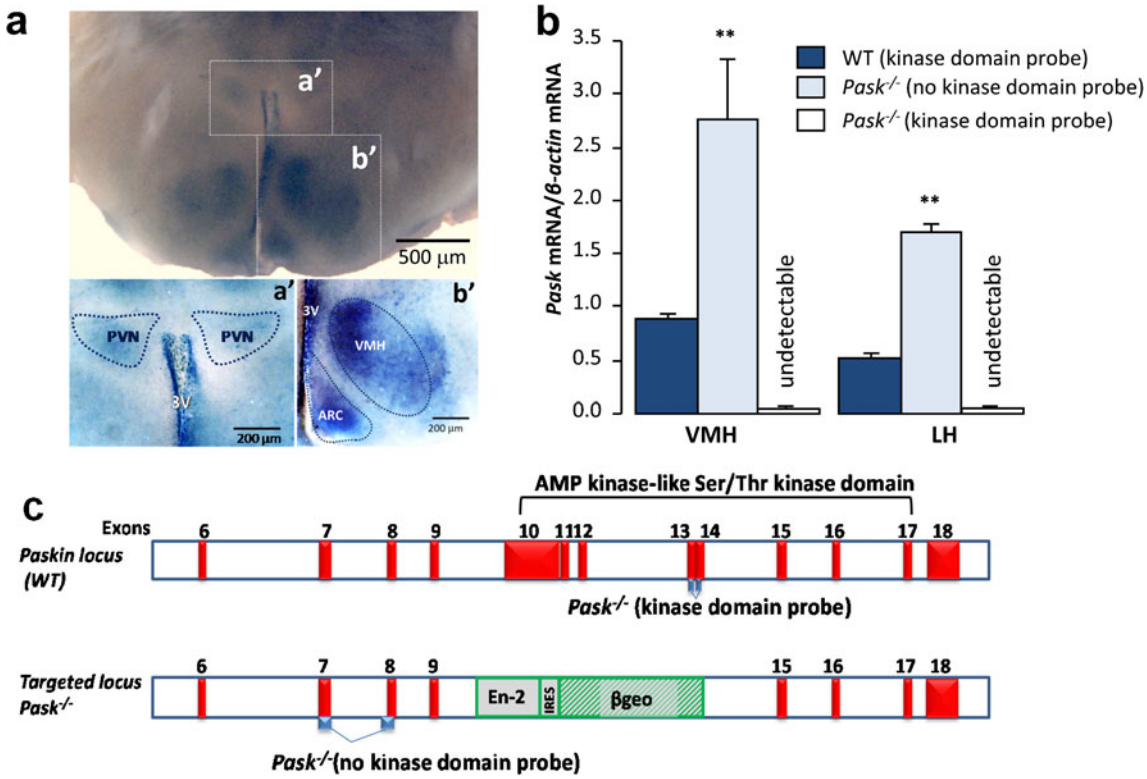

Fig. 1 PASK is expressed in VMH and LH of mice. **a** *Pask* expression pattern, revealed by X-gal staining of a 500-µm hypothalamic slice from $Pask^{-/-}$ mice. (*a'*) and (*b'*) show a higher magnification of boxed areas. *PVN* paraventricular nucleus, *VMH* ventromedial nucleus, *ARC* arcuate nucleus, *3 V* third ventricle. **b** *Pask* mRNA levels in VMH and LH from wild-type (WT) and PASK-deficient mice ($Pask^{-/-}$). The mRNA coding for PASK was detected by quantitative real-time PCR, using two different probes located 5′ to the targeted region or located in the targeted kinase domain, as indicated. The *bars* represent the means ± SEM of the ratios respect to *β-actin* of the values obtained from quantitative real-time PCR of three independent experiments carried out in duplicate. The values obtained in the VMH of the wild-type animals were considered as 1. $^{**}P<0.01$, WT vs. $Pask^{-/-}$. **c** Schematic representation of the deleted kinase domain in $Pask^{-/-}$ mice and the location of probes used in the quantitative real-time PCR

VMH in wild-type mice. The truncated *Pask* (exon 7 and 8) mRNA levels of PASK-deficient mice were also elevated in both hypothalamic areas (Fig. 3b).

Impaired Activation of AMPK and Acetyl-CoA Carboxylase in VMH and LH from PASK-Deficient Mice After Fasting or Re-feeding States

Hypothalamic metabolic sensors, AMPK and mTOR/S6K1 pathways, play a critical role in monitoring food intake, and we therefore checked their response to fasting/re-feeding situations in PASK-deficient mice. The activation of AMPK was checked using an anti-phospho-AMPKα (Thr172). The maximal AMPK activity was observed in the fasted state. Three hours of re-feeding after fasting reduced AMPK activity in VMH and LH areas by ~40 and 50 %, respectively, in wild-type mice (Fig. 4a). In contrast, AMPK activity was elevated in both fasted and re-fed states in hypothalamic areas in PASK-deficient mice (Fig. 4a). Increased AMPK activity after fasting was reversed by exendin-4 administration for 1 h, specifically in LH from wild-type mice. However, the exendin-4 effect on AMPK activity was lost in PASK-deficient mice (Fig. 4a). AMPK activation is associated with the enhanced phosphorylation of its downstream target acetyl-CoA carboxylase (ACC). The level of ACC phosphorylation in LH was also reduced after re-feeding in wild-type mice (Fig. 4b). The phospho-ACC levels were elevated in both fasted and re-fed states in hypothalamic areas in PASK-deficient mice (Fig. 4b).

Altered S6K1 Activation in VMH and LH from PASK-Deficient Mice Under Fasting or Re-feeding Conditions

The activation/inhibition of the mTOR/S6K1 pathway was determined. In fasted conditions or after 3 h of re-feeding, the amount of S6K1 was similar in VMH and LH in both PASK-deficient and wild-type mice. The activation of S6K1 was detected using anti-phospho-S6K1 (Thr389). Re-feeding increased S6K1 activity significantly in LH, and similar results were observed in VMH, although no significant differences were recorded (Fig. 5). However, S6K1 activity was enhanced in both

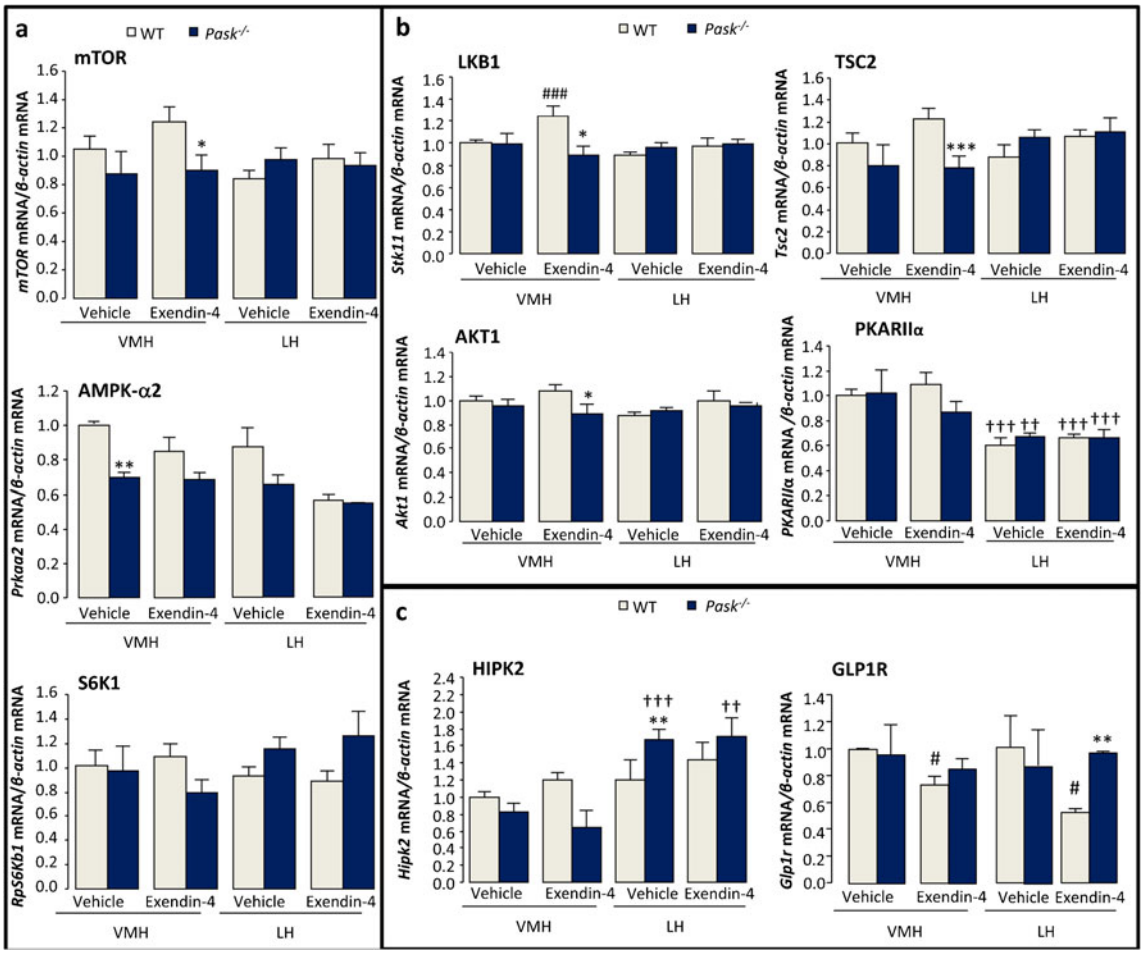

Fig. 2 Effects of PASK deficiency on the expression of several genes in VMH and LH**.** Quantitative real-time PCR was used to analyse the expression of several genes as shown (Online resource 1). The mRNA levels were quantified in VMH and LH from fasted C57Bl/6 wild-type and PASK-knockout mice in the presence or absence of exendin-4. The *bars* represent the ratios with respect to *β-actin* of the mRNA levels of the different genes. The value obtained in the VMH from wild-type mice (WT) treated with a vehicle without exendin-4 was taken as 1. Results are means ± SEM; n=3–4 animals per condition. *P<0.05, **P<0.01, ***P<0.001, WT vs. *Pask*$^{-/-}$; $^{\#}P$<0.05, $^{\#\#\#}P$<0.001, vehicle vs. exendin-4; $^{\dagger\dagger}P$<0.01, $^{\dagger\dagger\dagger}P$<0.001, VMH vs. LH

fasted and re-fed states in PASK-deficient mice (Fig. 5). Exendin-4 regulates hypothalamic S6K1 activation in vivo in fasted and re-fed wild-type mice. Nevertheless, the exendin-4 effect increased S6K1 activity in fasted

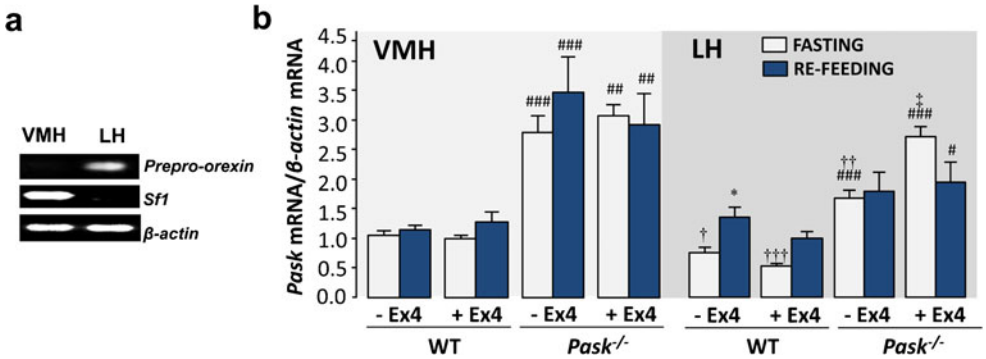

Fig. 3 Nutrients and exendin-4 modulate *Pask* gene expression in the VMH and LH from wild-type and PASK-deficient mice. **a** RT-PCR analysis of *Sf-1*, *prepro-orexin* and *β-actin* mRNA expression in the VMH and LH from wild-type mice. **b** *Pask* mRNA and *β-actin* levels were quantified by real-time RT-PCR in VMH and LH from fasted (FASTING) and re-fed (RE-FEEDING) wild-type (WT) and PASK-deficient (*Pask*$^{-/-}$) mice. The data were normalized to fasting untreated wild-type mice. Results are means ± SEM; n=3–4 animals per condition. *P<0.05, fasted vs. re-feed; $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001, WT vs. *Pask*$^{-/-}$; $^{\dagger}P$<0.05, $^{\dagger\dagger}P$<0.01, $^{\dagger\dagger\dagger}P$<0.001, VMH vs. LH; $^{\ddagger}P$<0.05, vehicle vs. exendin-4

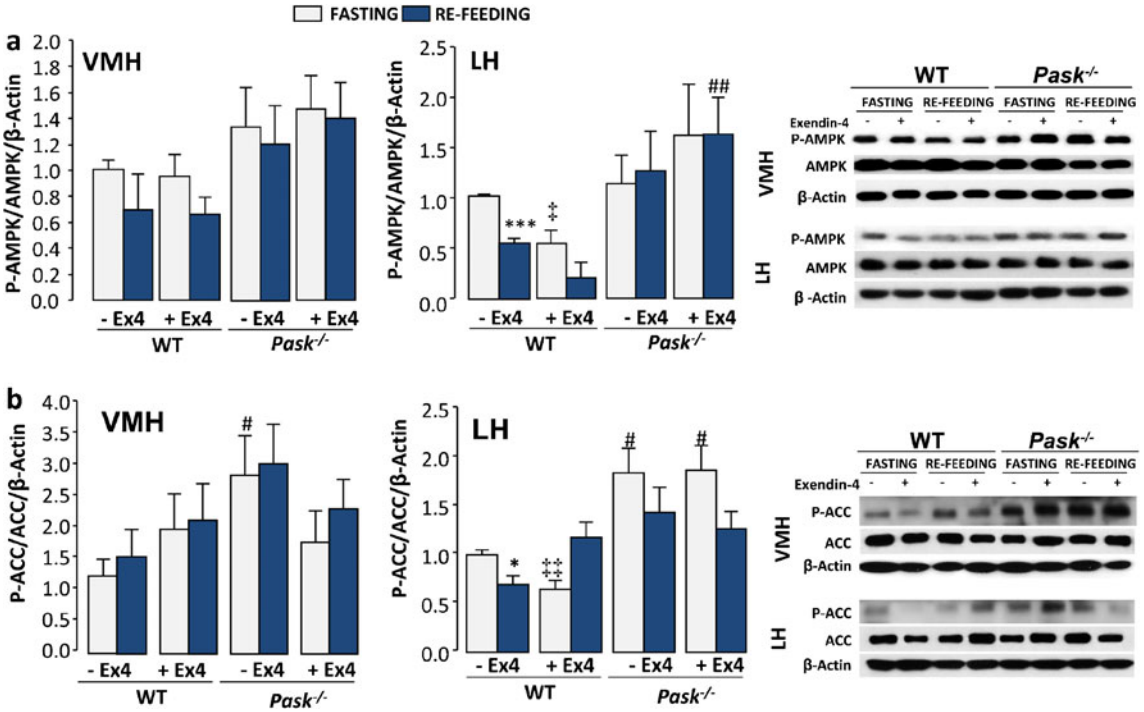

Fig. 4 Impaired effect of nutrients on AMPK and ACC activity in VMH and LH from *Pask*$^{-/-}$ mice. **a** Immunoblot analysis of phospho-AMPK (Thr172) (P-AMPK) and total AMPK (AMPK). **b** Immunoblot analysis of phospho-ACC (Ser-79) (P-ACC) and total ACC (ACC). The blots were reprobed for β-actin to normalize the results. Bar graphs represent means ± SEM of the densitometric values, β-actin and non-phosphorylated forms; n=3–4 animals per condition. Representative western blots of the graphs are also shown. The values were normalized by fasted wild-type untreated mice. *P<0.05, ***P<0.001, fasted vs. re-feed; $^{\#}P$<0.05, $^{\#\#}P$<0.01, WT vs. *Pask*$^{-/-}$; $^{\ddagger}P$<0.05, $^{\ddagger\ddagger}P$<0.01, vehicle vs. exendin-4

conditions and, in contrast, 3 h of re-feeding significantly decreased S6K1 activation in LH with similar results, although no significant differences were recorded in VMH from wild-type mice (Fig. 5). However, the exendin-4 effect on S6K1 activity was lost in PASK-deficient mice (Fig. 5).

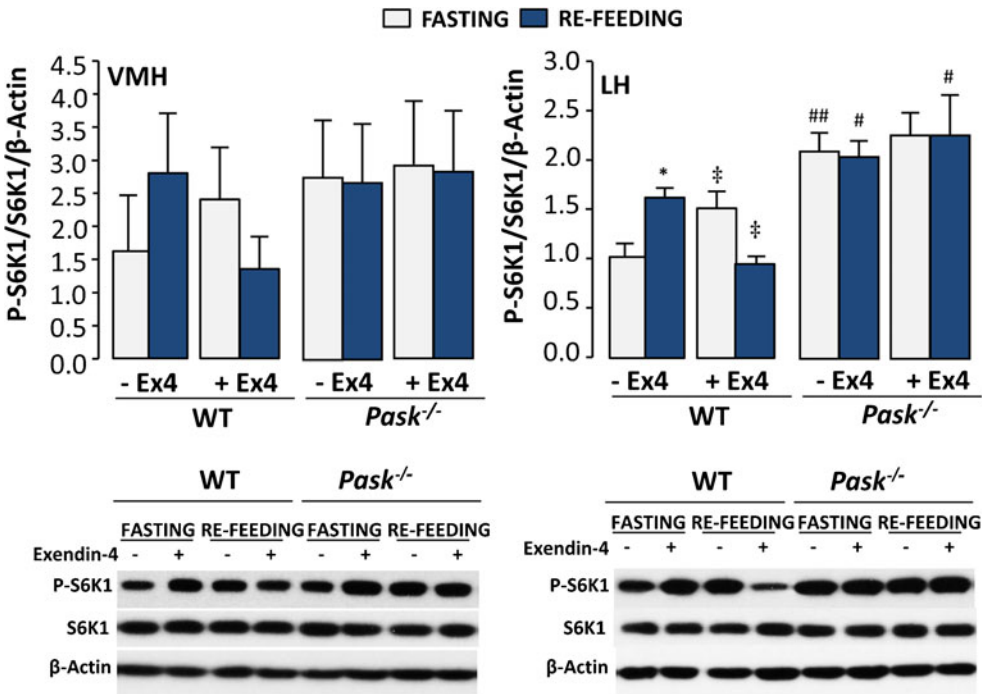

Fig. 5 Impaired effects of nutrients on S6K1 activation in VMH and LH from *Pask*$^{-/-}$ mice. Immunoblot analysis of phospho-S6K1 (Thr389) (P-S6K1) and total S6K1 (S6K1). The blots were reprobed for β-actin as a loading control. Bar graphs represent means ± SEM; n=3–4 of the densitometric values, expressed as the ratio with respect to β-actin and non-phosphorylated forms. Representative western blots of the graphs are also shown. The values were normalized by fasted WT untreated mice. *P<0.05, fasted vs. re-feed; $^{\#}P$<0.05, $^{\#\#}P$<0.01, WT vs. *Pask*$^{-/-}$; $^{\ddagger}P$<0.05, vehicle vs. exendin-4

PASK-Deficient Mouse Liver Has an Altered AMPK Response to Fasting/Re-feeding States

The activation/inactivation state of AMPK and S6K1 under fasting/re-feeding conditions was also checked in the liver to test the response of metabolic sensors in PASK-deficient mice in peripheral tissues. Our data showed no differences on AMPKα2 and S6K1 protein expression under the conditions analysed in both PASK-deficient and wild-type mice (Fig. 6a, b). The activation of AMPK decreased after re-feeding, and exendin-4 treatment reversed this effect in wild-type mice (Fig. 6a). In contrast, AMPK activity was enhanced after re-feeding in PASK-deficient mice (Fig. 6a). The maximal activation of S6K1 was observed after re-feeding, and the presence of exendin-4 reversed the effect in wild-type and PASK-deficient mice (Fig. 6b). Nevertheless, the S6K1 activation level was higher in PASK-deficient mice (Fig. 6b).

PASK-Deficient Mice Display Altered Feeding and Blood Glucose Levels in Response to Subcutaneous Exendin-4 Treatment

To check whether PASK is required for the exendin-4 anorexigenic effect on food intake, we tested the feeding response after subcutaneous administration of exendin-4 (250 ng/100 g body weight) in PASK-deficient mice. We have previously reported an anorexigenic effect of exendin-4 in lean and obese rats [24] and also suggested that this effect can be explained by the regulatory effect exendin-4 has on hypothalamic AMPK and S6K1 activities [25]. Food intake was similar in wild-type and PASK-deficient mice (Fig. 7a). The treatment with exendin-4 decreased food intake by more than 75 % after 1 to 2 h of re-feeding in control wild-type mice (Fig. 7a). However, the treatment with exendin-4 reduced food intake to 45 % in PASK-deficient mice (Fig. 7a). The exendin-4 treatment was more potent for inhibiting food intake in wild-type than in PASK-deficient mice, but only after 60 min of re-feeding (Fig. 7a).

Blood glucose levels were measured in *Pask*$^{-/-}$ mice fasted (24 and 48 h) and after re-feeding for 3 h in the absence or presence of exendin-4 (Fig. 7b). Glucose levels rise significantly after 3 h of re-feeding in both wild-type and *Pask*$^{-/-}$ mice, compared to fasted animals, although glucose circulating levels in fasted and re-fed animals were similar in both types of mice. Treatment with exendin-4 for 1 h significantly decreased the glucose levels in fasted and re-fed conditions. After re-feeding, the presence of exendin-4 decreased blood glucose levels by ~64 and ~42 % in wild-type and *Pask*$^{-/-}$ mice, respectively.

Serum insulin levels were similar in *Pask*$^{-/-}$ mice compared to wild-type controls in fasted and re-fed conditions (Table 2). The administration of exendin-4 for 1 h in fasted mice increased insulin levels in both wild-type and *Pask*$^{-/-}$ mice. By contrast, treatment with exendin-4 for 1 h in re-fed

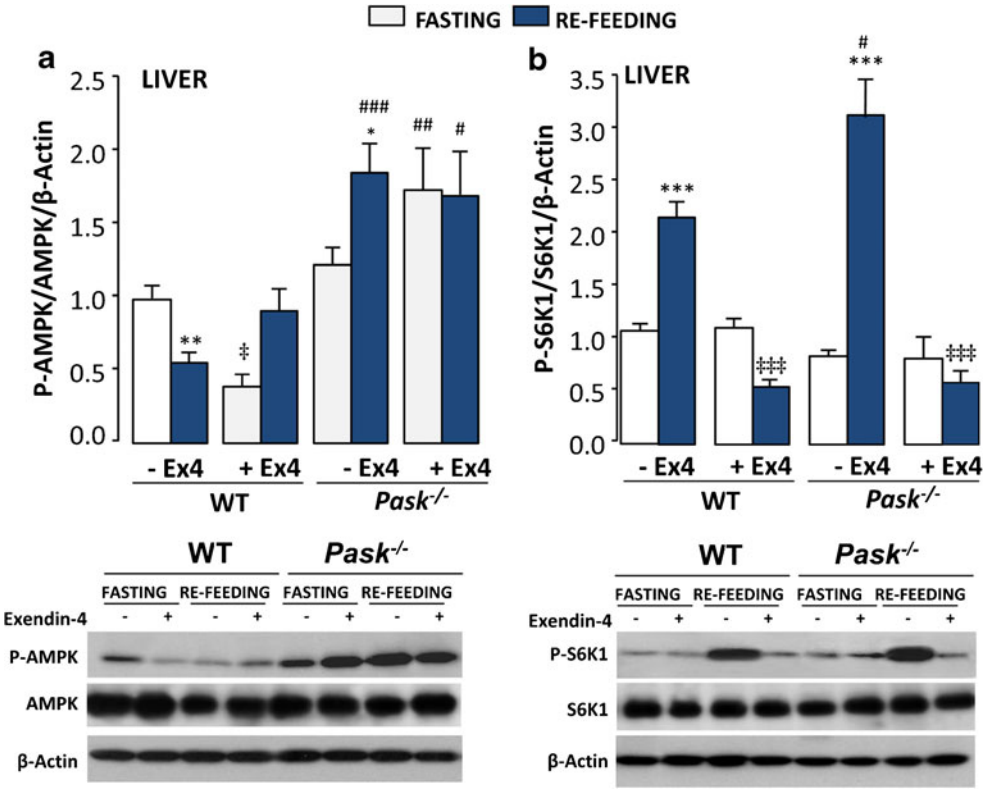

Fig. 6 Impaired effects of nutrients on AMPK activity in liver from *Pask*$^{-/-}$ mice. **a** Immunoblot analysis of phospho-AMPK (Thr172) (P-AMPK) and total AMPK (AMPK). **b** Immunoblot analysis of phospho-S6K1 (Thr389) (P-S6K1) and total S6K1 (S6K1). The blots were reprobed for β-actin to normalize the results. Bar graphs represent means ± SEM; n=3–4 of the densitometric values, expressed as the ratio with respect to β-actin and non-phosphorylated forms. Representative western blots of the graphs are also shown. The values were normalized by fasted WT untreated mice. $^{**}P<0.01$, $^{***}P<0.001$, fasted vs. re-feed; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, WT vs. *Pask*$^{-/-}$; $^{\ddagger}P<0.05$, $^{\ddagger\ddagger\ddagger}P<0.001$, vehicle vs. exendin-4

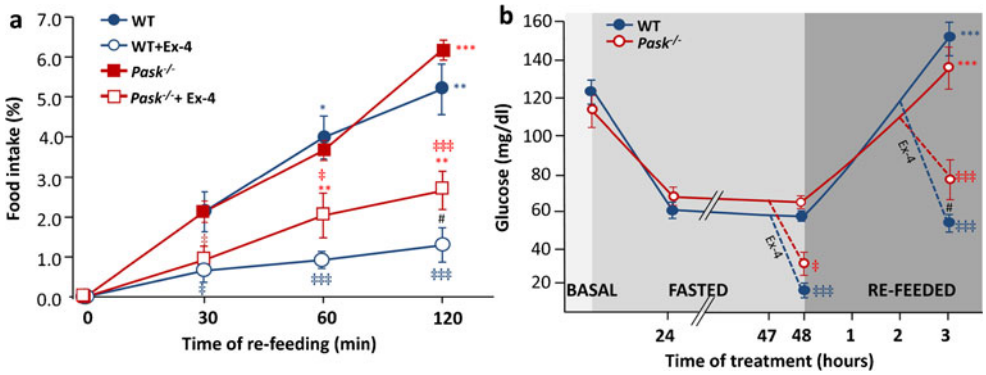

Fig. 7 Impaired effect of exendin-4 treatment in food intake and glucose circulating levels in PASK-deficient mice. **a** Effect of the subcutaneous administration of exendin-4 on food intake in fasted animals. Values are means ± SEM cumulative food intake during three consecutive intervals for six to eight determinations per group. *$P<0.05$, **$P<0.01$, re-feed 30 min vs. re-feed 60 and 120 min; $^{\#}P<0.05$, WT vs. $Pask^{-/-}$; $^{\ddagger}P<0.05$, $^{\ddagger\ddagger\ddagger}P<0.001$, vehicle vs. exendin-4. **b** Plasma glucose levels were measured in fasted mice (24 and 48 h) and after re-feeding for 3 h in the absence or presence of exendin-4 treatment for 1 h. $^{\#}P<0.05$, WT vs. $Pask^{-/-}$; $^{\ddagger}P<0.05$, $^{\ddagger\ddagger\ddagger}P<0.001$, vehicle vs. exendin-4. The determinations were made in eight animals per group

mice decreased serum insulin levels, and no significant differences were found between $Pask^{-/-}$ and wild-type mice. There is a concordance between the lower decrease of blood glucose levels after exendin-4 treatment in re-fed state, with the loss of anorexigenic power of exendin-4 in $Pask^{-/-}$ mice. However, no changes were observed in the incretin effect of this peptide in these mice with respect to wild-type ones.

Discussion

In this paper, we describe a role for PASK as a nutrient and hormonal sensor in hypothalamic areas involved in feeding behaviour with a different regulatory profile between VMH and LH. $Pask^{-/-}$ mice also have an impaired response of AMPK and mTOR/S6K1 pathways to fasting/re-feeding conditions in VMH, LH areas and the liver. Additionally, we focused our interest on the potential role of PASK as a mediator of GLP-1/exendin-4 effects.

Previous reports analysing PASK expression in response to glucose in pancreatic beta cells are contradictory. In MIN-6 cells, PASK expression is activated by the presence of high glucose concentrations and it is necessary for the transcription of the insulin gene mediated by glucose [30]. However, an analysis of pancreatic beta cells from mice in which the kinase domain has been removed (PASK knockout) showed that *Pask* mRNA expression did not respond to elevated glucose concentrations, and no differences in glucose-stimulated insulin production were found between both types of mice [31]. Later studies indicated that PASK-deficient mice are resistant to diet-induced obesity, with this effect being related to an increase in the metabolic rate in skeletal muscle, although there was no effect on the function of other metabolic sensors (AMPK, mTOR) [7].

However, *Pask* expression is not only restricted to peripheral tissues, as it is also expressed in the central nervous system, in hypothalamic areas involved in the control of food intake [23]. Our results obtained by X-gal staining indicate that PASK was located in several hypothalamic nuclei involved in the control of food intake such as VMH, PVN, ARC and lesser extended in LH. Nonetheless, the role of PASK acting on the central nervous system and the

Table 2 Blood insulin concentrations in mice injected sc with exendin-4 in fasted or re-feeding conditions

Feeding status	Time of exendin (10 nM) treatment (h)	Insulin concentrations (ng/ml)	
		WT	$Pask^{-/-}$
Basal	–	0.37±0.02 (n=3)	0.40±0.02 (n=3)
Fasted 48 h	–	0.22±0.05 (n=13)	0.19±0.06 (n=10)
	Exendin-4 (1 h)	0.77±0.32 (n=6)‡	1.01±0.41 (n=5)‡‡
Re-feeded 3 h	–	4.13±0.83 (n=10)***	6.10±0.99 (n=12)***
	Exendin-4 (1 h)	1.37±0.52 (n=8)‡	0.94±0.20 (n=5)‡‡

Male C57Bl/6 wild-type (WT) and PASK-deficient mice ($Pask^{-/-}$) were fasted for 48 h and then re-feeding for 3 h. Basal indicates mice housed in standard conditions, fed ad libitum, with no previous fasting period. Exendin-4 (250 ng/100 g body weight, Bachem) in saline or the saline vehicle alone (–) was subcutaneously (sc) injected during the last 1 h

*$P<0.05$; ***$P<0.001$ (fasted vs. re-feeded); ‡$P<0.05$; ‡‡$P<0.01$ (exendine-4 treatment vs. no exendine-4 treatment)

interrelationship with other metabolic brain sensors still remain poorly understood. The important role hypothalamic metabolic sensors play in controlling food intake and maintaining the energy balance throughout the body has been extensively studied in recent years. At least two kinds of glucose sensor neurons have been described in the brain: glucose-excited neurons are located mainly in the VMH and are excited by increased glucose levels in the extracellular space, while glucose-inhibited neurons (mainly present in the LH) are excited by decreases in glucose concentrations [32]. These two areas play different roles in the control of food intake and were defined as centres of satiety and hunger, respectively. Hypothalamic AMPK and mTOR/S6K1 pathways respond to changes in glucose and other nutrients in opposite ways, and their effects on the regulation of food intake can overlap. We have also recently reported that the antidiabetogenic and anorexigenic agent exendin-4 counteracts the activation/inactivation of AMPK and mTOR/S6K pathways in hypothalamic areas involved in feeding behaviour, such as VMH and LH, helping to balance the proper activation of these metabolic sensors in lean and obese rats [25].

Pask mRNA levels in LH were upregulated after re-feeding. However, we observed an elevated expression of truncated PASK mRNA levels in both VMH and LH in PASK-deficient mice and a lack of regulation under fasting or re-feeding conditions. We have previously reported that the *Pask* gene was regulated by glucose and the anorexigenic peptide GLP-1, which is released from intestinal L-cells in the presence of nutrient abundance and exerts a dual role as an incretin and anorexigenic peptide reporting the nutrient status to the brain. Thus, a high glucose concentration decreased *Pask* mRNA expression in VMH and LH from rat hypothalamic organotypic cultures. However, GLP-1 treatment at elevated glucose concentrations increased *Pask* mRNA in LH [23]. The effect of high glucose and GLP-1 in LH is consistent with the results found in vivo, taking into account that the re-feeding state must be characterized by both elevated glucose and GLP-1 levels released after eating.

Another interesting finding is that PASK deficiency alters the nutrient response of AMPK and mTOR/S6K1 pathways in hypothalamic areas involved in feeding behaviour. Our data show the activation of hypothalamic AMPK in fasted state compared to re-feeding conditions, with the opposite effect in the activation level of S6K1 in LH in wild-type mice. Similar data were observed in VMH, although the differences were not significant. These data are consistent with the results previously described in rodents [21, 25, 33, 34]. However, exendin-4 treatment in fasted animals induced the activation of both metabolic sensors AMPK and S6K1 in a similar way to the re-feed condition, although exendin-4 treatment decreased blood glucose levels. These data suggest that the exendin-4 effect prevails over the glucose regulatory effect. In contrast, the treatment with exendin-4 in re-feeding conditions decreased glucose circulating levels, and the activation of S6K1 also decreased significantly, although AMPK activity remained low. Although previous results indicated that there is no relationship between PASK deficiency and the activity of other metabolic sensors in the liver or muscle under the conditions studied [7], we observed that PASK deficiency impaired AMPK and S6K1 response to both the nutritional state and exendin-4 treatment in hypothalamic areas. In this way, AMPK and S6K1 showed an elevated activation state in fasted and re-fed conditions, respectively, in both LH and VMH. Additionally, in this study, we observed that the regulatory effect of exendin-4 in both AMPK and S6K1 was lost in PASK-deficient mice. A similar effect was observed in PASK knockdown neuroblastoma cells, suggesting that PASK is implicated in some GLP-1 actions [23]. We also observed that AMPK response to nutrients was altered in peripheral tissues as the liver in PASK-deficient mice. In fasted animals, the activation was similar to wild-type mice in accordance with the data reported by Hao et al. [7]. Nevertheless, the activation level of AMPK increased after re-feeding, and activation of S6K1 was also higher than in control mice. Then, AMPK and mTOR were both activated simultaneously after re-feeding while GSK3β was inhibited in the liver from PASK-deficient mice (data not shown). It is accepted that the activation of AMPK in the liver and muscle can cause a decrease in blood glucose and lipid levels [35]. The elevated AMPK activity may also explain the increased glucose and lipid oxidation previously reported [7].

We have also confirmed here that the anorexigenic effect of exendin-4 was partially inhibited and yet still observed in PASK-deficient mice. The anorexigenic effect of exendin-4 was reduced in PASK-deficient mice. This finding is also in accordance with the significantly higher blood glucose levels found after exendin-4 treatment in PASK-deficient mice. Thus, PASK-deficient mice record a higher blood glucose concentration and similar insulin levels after exendin-4 administration during the last hour. However, glucose and insulin circulating levels in fasted and re-fed conditions were not significantly altered in PASK-deficient mice, confirming previous results of food intake and those obtained with a glucose tolerance test [7]. Nevertheless, our data suggest that some of the exendin-4 effects are mediated through AMPK, mTOR/S6K1 and PASK pathways that are impaired, but other hypothalamic signalling pathways are still active in PASK-deficient mice.

The activation of mTOR in the hypothalamus has been related to anorexia [22, 34, 36, 37]. However, mice with a TSC1 deficiency in the hypothalamus and pro-opiomelanocortin (POMC) neurons, which promotes mTOR activation, develop an orexigenic response [38]. It has also been reported that the orexigenic effect of ghrelin, one of the intestinal peptides, is mediated by the hypothalamic mTOR

pathway [39]. Ghrelin also activates the AMPK and stimulates food intake [40, 41]. Both AMPK and mTOR/S6K1 pathways have opposite cellular effects for regulating metabolism and cell growth, for example. Thus, AMPK and mTOR respond to changes in glucose and other nutrients in opposite ways, and their effects on the regulation of food intake may overlap. Along these lines, previous data have reported interrelations between both routes. The activation of AMPK induces the inhibition of mTOR activity and also that of S6K1 phosphorylates while inhibiting the activity of AMPKα2 in the hypothalamus [20]. AMPK and mTOR in PASK-deficient mice are both activated simultaneously in the areas involved in the control of food intake, suggesting that the PASK function could be critical for maintaining both the regulation of the on/off activation of both pathways and the regulatory role of exendin-4 in food intake. Inoki et al. reported that inhibition of mTOR by AMPK activation required a coordinated phosphorylation of TSC2 by AMPK and GSK3β [42]. In accordance, we observed that GSK3β was more inhibited after re-feeding in liver from PASK-deficient mice (data not shown). These results would suggest one possible explanation for the lack of coordination between AMPK activation and mTOR/S6K1 inhibition in PASK-deficient mice although subsequent studies will be needed to clarify this hypothesis. The deregulation of these signalling pathways might be involved in the development of obesity and type 2 diabetes. A mutation in the *PASK* gene has been described in the early onset of diabetes, which modulates glucose-stimulated insulin secretion [43]. A decrease in PASK expression was also found in pancreatic islets from human with type 2 diabetes [8]. These data seem to contradict the resistance to the development of obesity previously described in PASK-deficient mice [7].

The importance of PASK and its relationship with diseases such as obesity and type 2 diabetes may also depend on its ability to regulate AMPK and mTOR/S6K1 activities, as altered responses of AMPK could also be involved in obesity and type 2 diabetes [44]. Several examples have been reported: the use of AMPK-knockout mice deficient in the catalytic subunit has shown that while AMPKα1$^{-/-}$ mice have no metabolic alterations, AMPKα2$^{-/-}$ mice are insulin-resistant, with no apparent changes in body weight or food intake [45–47]. Specific deletions of AMPKα2 in hypothalamic POMC neurons or in hypothalamic agouti-related peptide (AgRP) neurons modified the feeding behaviour and the maintenance of energy and body weight homeostasis. Thus, the AMPKα2 KO in POMC neurons developed obesity and lower energy expenditure [48]. By contrast, mice with an AMPKα2 deficiency in AgRP neurons developed an age-dependent lean phenotype [48]. Furthermore, it has also been reported that AMPKβ1-knockout mice had a lower food intake and reduced total body weight [49]. Neuronal protein tyrosine phosphatase 1B-deficient mice are resistant to diet-induced obesity, showing lower hypothalamic AMPK activation and higher energy expenditure [50]. S6K1-deficient mice are also resistant to diet-induced obesity [51]. In contrast, regarding PASK-deficient mice that are also resistant to diet-induced obesity, we observed that mTOR/S6K1 pathway is highly activated in the hypothalamus, and the AMPK is also activated, which furthermore contradicts the data reported on PASK knockdown by siRNA in cultured myoblasts that are posited to cause increased glucose and palmitate oxidation and elevated cellular ATP levels [7]. It also contradicts our data on PASK knockdown neuroblastoma cells in which we observed increased ATP levels and the loss of AMPK activation at low glucose levels [23].

The interpretation of the phenotypes in knockout mice is difficult due the redundancy of regulatory mechanisms. Several hypothalamic sensors detect nutrient levels, which could compensate and interrelate with each other, as well as the presence of many anorexigenic peptides that control their activation and inactivation in order to regulate feeding behaviour and energy homeostasis and maintain body weight. However, in vitro studies, where the compensatory mechanisms are limited, have revealed that PASK plays as a nutrient sensor in neuroblastoma and hypothalamic cells [23]. We also show here how PASK is involved in the control of the gene expression of several genes, such as *Ampkα2* and *Hipk2* and its role as a mediator of the effects of exendin-4 on the regulation of genes coding to LKB1, TSC2, AKT1, mTOR, eEF1-α1 and nNOS1. The effects of PASK deficiency were different in VMH and LH. These hypothalamic areas contain different distributions of neuron glucose responsive (GE) or glucose inhibited (GI) predominant in VMH and LH, respectively. These differences can also explain changes in the expression of PASK that was higher in VMH than that in LH. For this reason, we cannot rule out that PAKS deficiency may have different effects or may be compensated differently in both areas. In short, our results show that PASK is a nutrient sensor in hypothalamic areas involved in feeding behaviour, and the PASK function is decisive for maintaining the nutrient effect on hypothalamic AMPK and mTOR/S6K1 pathways. Furthermore, PASK is required for the correct signalling of GLP-1/exendin-4.

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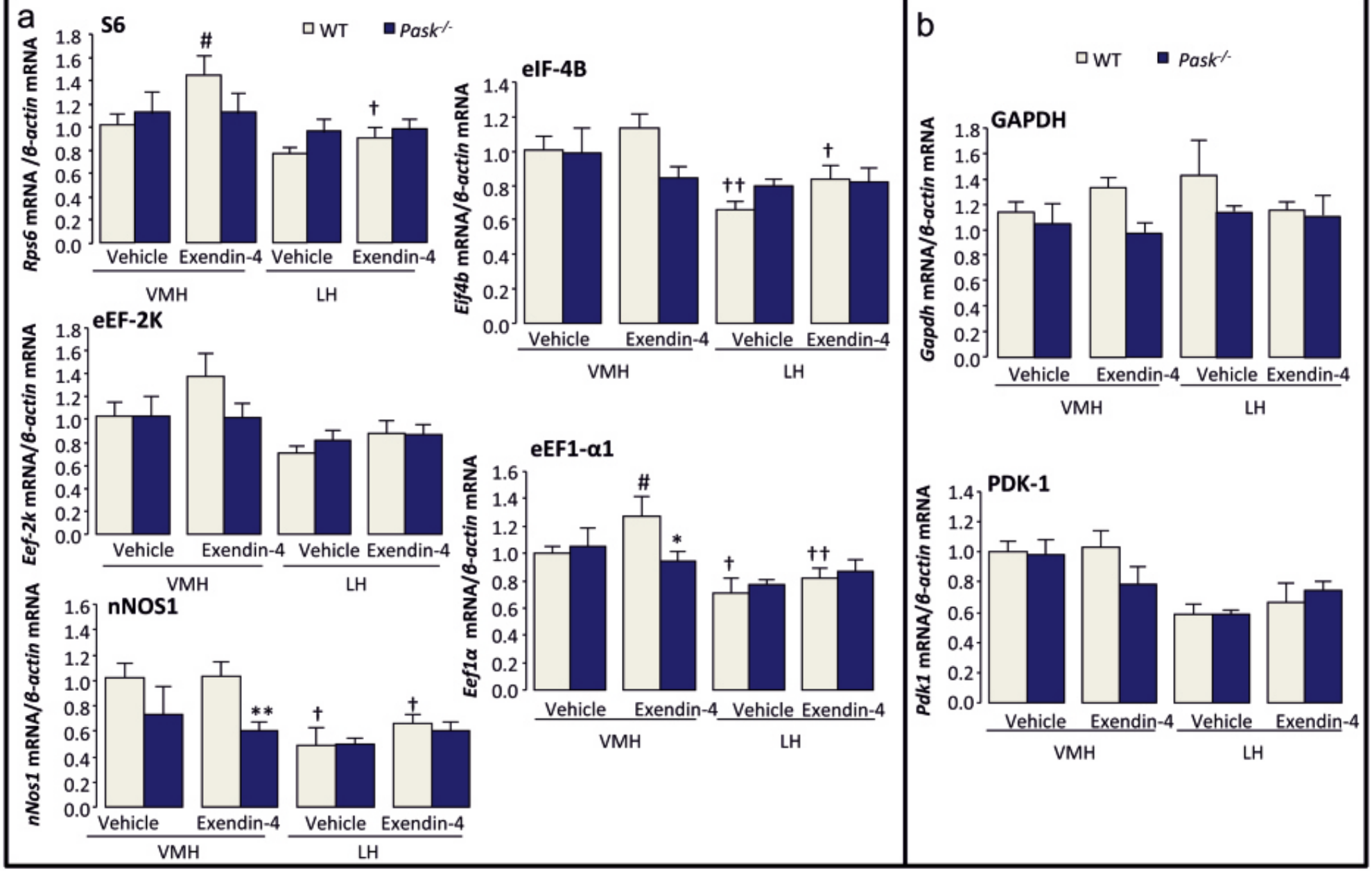
On line resource 1. Identification of probes used in the different gene expression assays

MULTIPLE TAQMAN® GENE EXPRESSION ASSAY

Gene Symbol	Gen Identification	Mouse probe identification (Taq Man®Assay)	Protein name
Rn 18s	19791	Hs99999901_s1	18S
Actb	11461	Hs99999903_m1	β-ACTIN
Pask	269224	Mm00435916_m1	PASK
Prkar2a	19087	Mm00435916_m1	PKA RIIα
Rps6kb1	72508	Mm01310033_m1	S6K1
Mtor	56717	Mm00444968_m1	mTOR
Eif4b	75705	Mm00778003_s1	eIF-4B
Rps6	20104	Mm02342456_g1	S6
Eef2k	13631	Mm00432996_m1	eEF-2K/CaMK
Eef1a1	13627	Mm01973893_m1	eEF1-α1
Stk11	20869	Mm00488470_m1	LKB1
Pdk1	228026	Mm00499855_m1	PDK1
Tsc2	22084	Mm00442004_m1	TSC2
Akt1	11651	Mm01331624_m1	AKT1
nNos1	18125	Mm00435175_m1	nNOS
Hipk2	15258	Mm00439329_m1	HIPK2
Gapdh	14433	Mm99999915_g1	GAPDH
REAL-TIME POLYMERASE CHAIN REACTION (TAQMAN® ASSAY)			
Gene Symbol	**Primers (Forward and reverse)**	**Taqman probe**	**Protein name**
Rat Prkaa2	5´-GTGGATCGCCAAATTA TGCA-3´ 5´-GGGAGGGTGCCACA GAGAA-3´	6-FAM-TAC AGC CTT CTT GAC ATG A-MGB	AMPKα2
Mouse Prkaa2		Mm 01264789_m1	AMPKα2
Rat Pask	5´-TGGACTGCAGTGGAGAA GGGA-3´ 5´-ATCCTCTAAAACCTTCT CCTTCTTA ATAAA-3´	6-FAM-ACA AAG AGA TGG TGG TGA A-MGB	PASK
POLYMERASE CHAIN REACTION			
Mouse *Glp-1r*		Mm 00445292_m1	GLP-1R
Mouse-*Rat Sf-1*	5´ –AGCTGCTGGTGTTGGACCA-3´ 5´-TCCAGTAACCAGCAGGATGCT-3		SF-1
Mouse *Prepro-orexin*	5´ –CTCAGACTCCTTGGGT ATTT-3´ 5´-CAATCCGGAGAGAGA TGGT -3´		PREPRO-OREXIN


a
S6
WT
Pask-/-
Rps6 mRNA /β-actin mRNA
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Vehicle
Exendin-4
Vehicle
Exendin-4
VMH
LH
eIF-4B
Eif4b mRNA/β-actin mRNA
††
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eEF-2K
Eef-2k mRNA/β-actin mRNA
eEF1-α1
Eef1α mRNA/β-actin mRNA
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nNos1 mRNA/β-actin mRNA
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b
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Pask-/-
GAPDH
Gapdh mRNA/β-actin mRNA
PDK-1
Pdk1 mRNA/β-actin mRNA